SNI

Standar Nasional Indonesia

SNI EN 455-3:2015
(Ditetapkan oleh BSN tahun 2020)

Sarung tangan medis sekali pakai - Bagian 3: Persyaratan dan pengujian untuk evaluasi biologis

Medical gloves for single use - Part 3: Requirements and testing for biological evaluation

(EN 455-3:2015, IDT, Eng)

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ICS 11.140

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Prakata

Standar Nasional Indonesia (SNI) EN 455-3:2015, dengan judul *Sarung tangan medis sekali pakai - Bagian 3: Persyaratan dan pengujian untuk evaluasi biologis (EN 455-3:2015, IDT, Eng)*, merupakan hasil adopsi identik dari standar EN 455-3:2015 *Medical gloves for single use - Part 3: Requirements and testing for biological evaluation*, dengan metode republikasi *reprint*, yang ditetapkan oleh BSN pada tahun 2020.

Standar ini disusun oleh Komite Teknis 13-09 Biosafety and Biosecurity dengan Badan Standardisasi Nasional (BSN) sebagai sekretariat Komite Teknis. Standar ini telah dibahas dalam rapat-rapat teknis, dan terakhir disepakati dalam rapat konsensus di Jakarta pada tanggal 17 April 2020 yang dihadiri oleh para pemangku kepentingan (*stakeholder*) terkait, yaitu perwakilan dari produsen, konsumen, pakar dan pemerintah, serta perwakilan dari lembaga penguji, asosiasi, perguruan tinggi, pakar serta instansi terkait.

Standar ini telah melalui tahap jajak pendapat pada tanggal 11 Mei 2020 sampai dengan 30 Mei 2020 dengan hasil akhir disetujui menjadi SNI.

Dalam standar ini digunakan kosa kata yang mempunyai maksud tertentu, yaitu:

- “harus” yang artinya disyaratkan.
- “sebaiknya” yang artinya direkomendasikan.

Perlu diperhatikan bahwa kemungkinan beberapa unsur dari dokumen standar ini dapat berupa hak paten. Badan Standardisasi Nasional tidak bertanggungjawab untuk pengidentifikasian salah satu atau seluruh

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Pendahuluan

Adverse reactions to proteins in latex products have been reported over several years in variable rates of prevalence. Additionally, adverse reactions due to chemicals, lubricants, sterilization residues, pyrogens or other residues are described in the scientific literature. Adverse reactions are most often reported due to gloves made from natural rubber latex, but some of the reactions can also be seen due to gloves made from synthetic polymers.

EN ISO 10993 specifies requirements and test methods for biological evaluation of medical devices. However it does not specifically address adverse reactions that can result from the use of medical gloves (e.g, immediate type allergies). These adverse reactions occur to specific allergens that can be present in gloves. Several factors contribute to the risk of reaction:

a) the duration and frequency of skin contact with gloves;
b) the exposure to the allergens through direct contact to mucosa and skin (especially when not intact) and by inhalation of particles;
c) the occlusive nature of the glove/skin interaction during glove use.

This part of EN 455 gives requirements and test methods for evaluation of the biological safety of medical gloves as part of a risk management process, in accordance with EN ISO 10993.

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Sarung tangan medis sekali pakai - Bagian 3: Persyaratan dan pengujian untuk evauasi biologis

1 Scope

This part of EN 455 specifies requirements for the evaluation of biological safety for medical gloves for single use. It gives requirements for labelling and the disclosure of information relevant to the test methods used.

2 Normative reference

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 1041:2008+A1:2013, Information supplied by the manufacturer of medical devices

EN ISO 10993-1:2009, Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process (ISO 10993-1:2009)

EN ISO 10993-5:2009, Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (ISO 10993-5:2009)

EN ISO 10993-10:2013, Biological evaluation of medical devices -Part 10: Tests for irritation and skin sensitization (ISO 10993-10:2010)

EN ISO 14971:2012, Medical devices - Application of risk management to medical devices (ISO 14971:2007, Corrected version 2007-10-01)

EN ISO 15223-1:2012, Medical devices - Symbols to be used with medical device labels, labelling and information to be supplied - Part 1: General requirements (ISO 15223-1:2012)

EN ISO 21171:2006, Medical gloves - Determination of removable surface powder (ISO 21171:2006)

European Pharmacopoeia, General chapter 2.6.14 Bacterial Endotoxins: publisher EDQM - Council of Europe; 7 allée Kastner, CS 30026, F-67081 Strasbourg; France http://www.edqm.eu/

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
chemicals
substances added or formed during any step of the manufacturing process or in storage which may be available in the final product

Note 1 to entry: These can include lubricants, chemical coatings and sterilizing agents. Several chemical ingredients are commonly used during processing of gloves, some of them are known to cause type IV allergic reactions. The type and amount of residual chemicals added and finally present are variable.

3.2
endotoxins
lipo-polysaccharides originating from the outer cell-membrane of Gram-negative bacteria
Note 1 to entry: Endotoxins are one type of pyrogen. Sources of endotoxins can include bacterial contamination of the raw materials, especially the process water used during manufacturing and manual handling of the gloves.

3.3
powder
all water insoluble material on the surface of a glove that is removed by washing under the conditions of the test

[SOURCE: EN ISO 21171:2006, 3.1]

Note 1 to entry: This includes both deliberately added powder and other processing aids or materials accidentally present which may be readily detached from the surface of the glove. For the purpose of this European Standard any glove containing 2 mg or less powder is a powder-free glove and more than 2 mg is a powdered glove (for requirement see 4.4.).

3.4
process limit
highest value likely to be encountered for a validated manufacturing process

3.5
proteins, allergenic
proteins capable of causing a type I allergic reaction

3.6
proteins, leachable
aqueous proteins and peptides extractable from the final product

3.7
pyrogens
substances creating fever in rabbits which can be related to fever and other adverse reactions in humans

4 Requirements

4.1 General

EN ISO 10993-1:2009 describes the general principles governing the biological evaluation of medical devices and shall be used to select the appropriate tests as described in other parts of the series. Based on EN ISO 10993-1:2009 medical gloves are classified as limited contact duration surface devices and require compliance to EN ISO 10993-5:2009 and EN ISO 10993-10:2013.

The classification of medical gloves according to EN ISO 10993-1:2009 should not be confused with the definitions provided in the medical device directives for these products.

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A risk management process in accordance with EN ISO 14971:2012 shall be established.

4.2 Chemicals

Gloves shall not be dressed with talcum powder (magnesium silicate).

The manufacturer shall disclose, upon request, a list of chemical ingredients either added during manufacturing or already known to be present in the product such as accelerators, antioxidants and biocides that are known to cause adverse health effects based on current data.

Upon request the manufacturer shall provide evidence of the steps taken to reduce the risk to the end-user of exposure to chemicals used in the manufacturing process which, based on current data, are known to cause adverse health effects.

Manufacturers may only declare the absence of a substance if the substance is not used in any part of the manufacturing process. No compounds shall be used in the manufacture of the product that is known to form a substance that is subject of such a declaration.

4.3 Endotoxins

The manufacturer shall monitor the endotoxin contamination of sterile gloves using the test method specified in 5.1 if the gloves are labelled with 'low endotoxin content'. For such labelled gloves the endotoxin content shall not exceed the limit of 20 endotoxin units per pair of gloves.

4.4 Powder-free gloves

For powder-free gloves the total quantity of powder residues determined according to the test method under 5.2 shall not exceed 2 mg per glove. Any glove containing more than 2 mg powder is a powdered glove.

4.5 Proteins, leachable

The manufacturer shall strive to minimize the leachable protein level.

The manufacturer shall monitor the process limit of leachable protein in the finished gloves containing natural rubber latex by the method specified in 5.3 and described in Annex A. The documentation of these results shall be retained. The results of the test and applied test method shall be made available on request.

NOTE Proteins, allergenic: This European Standard specifies a method measuring a broad approximation for the allergen content, e.g. leachable proteins. There is no direct correlation between leachable proteins and allergen content. Quantitative methods to measure allergenic proteins are, described in Annex B.

4.6 Labelling

In addition to the labelling specified in EN 1041:2008+A1:2013 and the relevant symbols given in EN ISO 15223-1:2012, the following requirements apply:

a) medical gloves containing natural rubber latex shall be labelled on the packaging of at least the smallest packaging unit with the EN ISO 15223-1:2012 symbol for latex (reference number 5.4.5).

The labelling shall include the following or equivalent warning statement together with the symbol (Product) contains natural rubber latex which may cause allergic reactions, including anaphylactic responses;

b) the labelling shall include a prominent indication of whether the glove is powdered or powder-free;
c) sterile powdered gloves shall be labelled with the following or equivalent: 'CAUTION: Surface powder shall be removed aseptically prior to undertaking operative procedures in order to minimize the risk of adverse tissue reactions;

NOTE 1 This caution statement can be given on the inner wrapping.

d) for any medical glove containing natural rubber latex the product labelling shall not include:
 - any term suggesting relative safety, such as low allergenicity, hypoallergenicity or low protein;
 - any unjustified indication of the presence of allergens;
e) if the manufacturer labels the gloves with the protein content, the process limit, measured as specified in 5.3 shall be given.

NOTE 2 This does not allow a protein labelling claim below 50 µg/g. Lower claims are not considered to be reliable given the expected process variation in manufacture and inter-laboratory testing.

5 Test methods

5.1 Endotoxins

Except where non-removable interferences in the Limulus Amoebocyte Lysate (LAL) procedures are present, selection, validation and use of technique shall be as described in the European Pharmacopoeia, Monograph 2.6.14, "Bacterial Endotoxins". The results shall be expressed in endotoxin units (E.U.) per pair of gloves.

NOTE 1 Where non-removable interferences in the LAL procedure are present, the bacterial endotoxin level cannot be accurately measured.

The minimum number of pairs of gloves recommended to be tested in relation to the number of items in the batch are two pairs of gloves for a batch size under thirty, three pairs of gloves for a batch size thirty to one hundred, and 3 % of a batch above size one hundred, up to a maximum of ten pairs of gloves per batch.

The outside surface of a pair of gloves is extracted with 40 ml of endotoxin-free water (Water LAL, European Pharmacopoeia, for not less than 40 min and not more than 60 min at a temperature between 37 °C and 40 °C in a way to ensure that all surfaces come into contact with the extraction medium. The extract is centrifuged, if necessary, for 15 min at 2 000 *g* to remove particles after which the liquid component is decanted and tested for endotoxin immediately afterwards.

NOTE 2 Other methods for the analysis of endotoxins exist and these can be used for routine quality control purposes provided they have been validated and a correlation established against the reference method specified in this European Standard.

5.2 Powder

The test method for the determination of powder residues described in EN ISO 21171:2006, Clauses 7 and 9 shall be used.

5.3 Proteins, leachable

The test method for the analytical determination of leachable protein shall be the modified Lowry method given in Annex A or a suitably validated method which has been correlated against the modified Lowry method.

NOTE 1 An example of a validated analytical method is given in Annex C.

NOTE 2 The immunological methods in Annex B are currently not validated against the modified Lowry method but may be correlated to clinical response data.

6 Test report

The test report shall include at least the following information:
— reference to this part of EN 455;
— the type of gloves and the manufacturing batch code;
— the name and address of the manufacturer or distributor and of the test laboratory, if different;
— the date of the testing performed;
— the description of the test method applied;
— the test results.

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Annex A
(normative)
Method for the determination of aqueous extractable proteins in natural rubber gloves using the modified Lowry assay

A.1 Scope

This method is for the determination of the amount of aqueous extractable proteins in gloves for medical use made from natural rubber (NR). It has been validated during inter-laboratory tests. The lower quantification limit is approximately 10 µg protein per g of glove (i.e. 2 µg protein per ml of extract) depending on the glove weight.

Chemicals such as surfactants, accelerators and antioxidants added to the NR latex during the manufacture of the gloves can interfere with the colour development during the determination, some materials may reduce colour development while others can increase it. If the test method yields results that appear erroneous due to interferants, then any validated amino acid analysis method can be used (as an example see the method given in Annex C).

Persons using this method should be familiar with normal laboratory practice.

NOTE This method does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

A.2 Principle

Water soluble proteins are extracted into a buffer solution and then precipitated with acids in the presence of sodium deoxycholate to concentrate them and to separate them from water soluble substances which may interfere with the determination. The precipitated proteins are redissolved in alkali and quantified colorimetrically by a modified Lowry method. The assay is based on the reaction of proteins with copper and Folin reagent in an alkaline medium to give a characteristic blue colour. Spectrophotometric measurements are performed at a fixed wavelength in the range 600 nm to 750 nm.

A.3 Reagents

A.3.1 General

Wherever water is called for, double distilled water or water of equivalent quality should be used. All other reagents should be of analytical quality.

A.3.2 Extractant

A.3.2.1 N-tris-[Hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES), hemisodium salt.

A.3.2.2 Extraction buffer, 0,1 M, prepared by dissolving 24 g TES (A.3.2.1) in 1 l water. Any equivalent buffering system can be used provided the solution has sufficient buffering capacity to hold a pH of 7,4 ± 0,2 in the glove extracts.

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Prepare a sufficient quantity for the glove extraction (A.6.2), the preparation of the protein standard solutions (A.6.3.2) and the blank.

A.3.2.3 Dye solution, Bromophenol blue, sodium salt solution, prepared by dissolving 100 mg bromophenol blue in 1 l of water. Prepare a fresh solution every four weeks.

A.3.3 Lowry protein assay reagents

NOTE Reagents can either be prepared from off-shelf chemicals [1] or be purchased as commercial kits. The method for this European Standard was validated with a commercial kit. [1)]

A.3.3.1 Reagent A, Copper reagent (alkaline copper tartrate or copper citrate solution).

A.3.3.2 Reagent B, Diluted Folin reagent.

A.3.4 Sodium hydroxide, 0,1 M aqueous solution.

A.3.5 Sodium deoxycholate (DOC), 3,47 mM, prepared by dissolving 0,15 g sodium deoxycholate in water and diluting with water to 100 ml. Do not use this solution more than four weeks after it has been prepared.

A.3.6 Trichloroacetic acid (TCA), 4,4 mM in water, prepared by dissolving 72 g TCA in water and diluting with water to 100 ml.

A.3.7 Phosphotungstic acid (PTA), prepared by dissolving 72 g PTA in water and diluting with water to 100 ml. Do not use this solution more than four weeks after it has been prepared.

A.3.8 Ovalbumin, from chicken egg [2)] lyophilized, salt-free.

A.4 Apparatus

A.4.1 Synthetic gloves, powder-free.

A.4.2 Centrifuge, capable of reaching at least 6000 *g.*

A.4.3 Centrifuge tubes, 30 ml or 50 ml polypropylene tubes with a low protein binding capacity of 10 µg per tube or less. Do not use glass equipment because of surface absorption of proteins.

NOTE A method for the determination of protein binding capacity is described in A.5.

A.4.4 Filter units, single use, with 0,22 µm pore size and a low protein binding capacity of 10 µg per filter or less.

NOTE A method for the determination of protein binding capacity is described in A.5.

1) Lowry Micro DC Protein Assay Kit (catalogue number 500-0116), available from BioRad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA 9456547, USA. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

2) This ovalbumin is prepared from fresh chicken egg whites by ammonium sulfate fractionation and repeated crystallisation at pH 4,5; for example Sigma A 5503, chicken egg albumin, Grade V, available from Sigma Chemical Co. P.0. Box 14506, St Louis, MO 63178, USA is suitable. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

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A.4.5 Syringes, disposable, 20 ml, made of polyethylene or polypropylene.

A.4.6 Micro tubes, 2 ml, made of polypropylene.

A.4.7 Quartz cuvette, of 10 mm path length.

A.4.8 Microtitre plate, with 96 wells, flat bottomed, made of polystyrene, or disposable cuvettes (A.4.9).

A.4.9 Disposable cuvettes, 1,5 ml semi-micro, 10 mm path length, made of polystyrene.

A.4.10 Microplate reader, operating at a wavelength in the range 600 nm to 750 nm.

A.4.11 Spectrophotometer, operating in the wavelength range 230 nm to 750 nm.

A.4.12 Vortex mixer.

A.4.13 Micropipettes, with disposable polypropylene tips.

A.4.14 Clamps, for sealing gloves watertight during extraction. Pairs of aluminium bars lined with foam rubber and which can be screwed together (see Figure A.1) or 170 mm long plastic clips for haemodialysis are suggested.

A.4.15 Shaker.

A.5 Measurement of protein binding capacity

A.5.1 General

Single use polypropylene equipment (which is known to have a low protein binding capacity) is recommended for use throughout. The protein binding capacity shall be checked by the following methods before using centrifuge tubes or filter units from a new batch. The test shall be carried out within one day.

A.5.2 Protein binding capacity of centrifuge tubes

A.5.2.1 Prepare in a centrifuge tube (A.4.3) 30 ml of a reference solution containing 10 µg/ml ovalbumin by dilution of the protein stock solution (A.6.3.1) with the extraction buffer (A.3.2.2).

A.5.2.2 Transfer 10 ml test portions of the ovalbumin solution (A.5.2.1) to each of two fresh centrifuge tubes and shake the tubes on a shaker (A.4.15) ensuring that the whole surface is wetted by the solution. After 30 min transfer the solutions to a further two tubes and shake them. Repeat the procedure until each of the 10 ml portions has been exposed to five tubes. Store the remaining test solutions.

A.5.2.3 Determine the concentration of the protein in the reference solution and two test solutions in triplicate using the method given in A.6.4 to A.6.6.

A.5.2.4 Calculate the average absorbed ovalbumin from the expression:

$$O = \frac{10(R - T)}{5}$$

$$=2\ (R\text{-}T)$$

where

O is the absorbed ovalbumin in µg/tube;
R is the mean of three determinations of the ovalbumin content of the reference solution in µg/ml;
T is the mean ovalbumin content of the test solution after passage through the tubes (i.e. the mean of six values) in µg/tube.

The value for the absorbed albumin (O) shall be less than 10 µg per tube, otherwise the tubes are unsuitable for the determination.

A.5.3 Protein binding capacity of filter units

A.5.3.1 Prepare in a centrifuge tube (A.4.3) 30 ml of a reference solution containing 10 µg/ml ovalbumin by dilution of the protein stock solution (A.6.3.1) with the extraction buffer (A.3.2.2).

A.5.3.2 Prepare two stacks of five filter units (A.4.4). Filter 10 ml of the reference solution through each filter stack into a centrifuge tube (A.4.3).

A.5.3.3 Determine the protein content of the reference solution and the two test solutions in triplicate using the method given in A.6.4 to A.6.6.

A.5.3.4 Calculate the average absorbed ovalbumin from the expression:

$$O = \frac{10(R - T)}{5}$$

$$=2\ (R\text{-}T)$$

where

O is the absorbed ovalbumin in µg/tube;
R is the mean of three determinations of the ovalbumin content of the reference solution in µg/ml;
T is the mean ovalbumin content of the test solution after passage through the filter units (i.e. the mean of six values) in µg/tube.

The value for the absorbed albumin (*O*) shall be less than 10 µg per filter, otherwise the filters are unsuitable for the determination.

A.6 Procedure

A.6.1 General

The procedure involves the extraction of the gloves followed by purification and concentration of the extract by a factor of five. The determination on the extract is performed by reference to a calibration curve prepared using standard protein solutions which have been concentrated in the same manner.

The extraction procedure employed is one in which the inside of one glove and the outside of a second glove are extracted simultaneously. It allows the extraction volume to be minimized at 25 ml and avoids any loss of proteins to container surfaces because the extraction buffer is only exposed to the gloves.

NOTE Alternative extraction procedures may be used if they are validated against this method. A round robin test by selected laboratories in Europe and in the USA revealed equivalent results with the ASTM Standard D5712:1995 [**2**] when extracting cut pieces of gloves for 2 h at 25 °C in TES buffer pH 7,4.

A.6.2 Extraction procedure

A.6.2.1 Use synthetic gloves (A.4.1) to handle the glove samples used for the extraction. Take eight glove specimens of the same size and the same lot and separate them into four pairs. In the case of hand-specific gloves, choose four right-handed and four left-handed samples and separate them into two right-handed and two left-handed pairs.

Mark the cuff of one glove of each pair at a point (200 ± 10) mm from the tip of the middle finger and weigh the glove ($m1$) to the nearest 0,1 g. For each pair, insert the second glove inside the marked one so that they fit together as shown in Figure A.1 a).

NOTE The method for introducing one glove into the other is not of critical importance provided the gloves are manipulated as little as possible. One way it can be achieved is by sliding a rod in the thumb and the little finger of the inner glove to help to introduce them into the corresponding fingers of the second glove. Use the rods to insert the three other fingers.

A.6.2.2 Pour into the inner glove a sufficient quantity of dye solution (A.3.2.3) to fill all five fingers. Introduce 25 ml extraction buffer (A.3.2.2) at (25 ± 5) °C between inner and outer gloves. For larger gloves this volume may be increased to a maximum of 50 ml. Remove most air bubbles and seal the gloves with the clamp (A.4.14) at the 20 cm mark so as to produce a watertight seal as shown in Figure A.1 b).

A.6.2.3 Fix the gloves to the shaker (A.4.15) and shake for (120 ± 5) min at (25 ± 5) °C.

A.6.2.4 Remove the clamp and separate the gloves carefully. Take care not to contaminate the extract with the dye solution. If the extract is coloured blue, it shall be discarded and the extraction repeated with fresh gloves.

A.6.2.5 Carefully transfer the extract into a centrifuge tube (A.4.3) and clarify the extract either by centrifugation at not less than 2000 *g* for 15 min or filtration through a single use filter unit (A.4.4), or a combination of both as appropriate. Either store the resulting clear solution refrigerated at 2 °C to 8 °C and carry out the determination within 48 h or alternatively freeze aliquots of the solution at -18 °C or lower for a period not exceeding 2 months before analysis.

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A.6.2.6 Cut the section of the cuff above the 20 cm mark from the extracted outer glove, wipe the liquid off the surface with tissue, allow to dry at room temperature and weigh it to the nearest 0,1 g (*m*2). Calculate the mass (*m*) of the extracted part of the glove as follows:

$$m = m_1 - m_2$$

A.6.3 Protein standard

A.6.3.1 Stock protein solution

Prepare a solution of ovalbumin (A.3.8) with a nominal concentration of 1 mg/ml by dissolving 25 mg ovalbumin in 25 ml extraction buffer (A.3.2.2). Filter the solution through a 0,22 µm filter (A.4.4) and determine the true concentration of ovalbumin by using an UV spectrophotometer to measure the absorbance at 280 nm using a quartz cuvette (A.4.7). If the absorbance is divided by 0,715 [3)] it will give the exact concentration in mg/ml. The solution is stable for 2 days under refrigeration or for 2 months frozen at -18 °C. Thawing requires heating to 45 °C for 15 min.

A.6.3.2 Protein standard solutions

Prepare serial dilutions of the protein stock solution (A.6.3.1) using the extraction buffer (A.3.2.2), to make solutions with nominal concentrations of about 100 µg/ml, 50 µg/ml, 20 µg/ml, 10 µg/ml, 5 µg/ml and 2 µg/ml. Use extraction buffer as a blank. The solutions are stable for 2 days refrigerated or for 2 months frozen at -18 °C. Thawing requires heating to 45 °C for 15 min.

A.6.4 Precipitation and concentration of protein

A.6.4.1 Carry out the procedure in duplicate at (25 ± 5) °C.

A.6.4.2 Accurately transfer 1 ml each of the blank, protein standard solutions (A.6.3.2) and the four glove extracts (A.6.2.5) into micro tubes (A.4.6). Add 0,1 ml of DOC (A.3.5), mix by vortexing and allow to stand for 10 min. Add 0,1 ml of TCA (A.3.6) and 0,1 ml PTA (A.3.7), mix by vortexing and allow to stand for a further 30 min.

A.6.4.3 Centrifuge at 6000 *g* for 15 min. Decant the supernatant liquid and drain for 5 min by inverting each centrifuge tube on an absorbent paper.

A.6.4.4 Add 0,2 ml of 0,1 M sodium hydroxide solution (A.3.4) to each tube including the blank. Mix on a vortex mixer to re-dissolve the precipitated proteins. Ensure that the proteins are completely re-dissolved to a clear solution. Depending on the glove this sometimes needs an overnight standing refrigerated at (5 ± 3) °C. If any precipitate remains, add a further, measured quantity of the sodium hydroxide solution by 0,2 ml increments up to a total of 1 ml and use a 0,2 ml aliquot for subsequent steps. It can be useful to dilute the extract of such samples prior to precipitation.

NOTE The process of concentrating the protein by precipitation and re-dissolving is intended to purify the protein and to rid it of interferants. It is inevitable that during this process a certain amount of protein is lost and it is assumed for purposes of the test that the same percentage will be lost from the protein standard solutions as from the test sample extracts. However, the loss should be kept to the minimum as gross losses would not be reproducible.

3) Assuming a molecular weight of 43000 D and a molar extinction of 30745 at 280 nm and pH 7,4, the extinction of 1 mg/ml ovalbumin in 0,1 M TES buffer pH 7,4 is 0,715 using a light path of 1 cm [3].

A.6.5 Colour development

A.6.5.1 The method described here is adapted to the commercial kit used for validation. Other kits or reagents prepared from off-shelf chemicals can require other volumes and incubation times.

A.6.5.2 Add 0,125 ml Reagent A (A.3.3.1) into each micro tube containing the re-dissolved protein solutions including the blank. Mix well. Add 1 ml Reagent B (A.3.3.2), cap the tube, vortex and allow the colour to develop fully for 30 min. Should precipitation occur at this stage, centrifuge or filter to remove the precipitate before measuring the absorbance.

A.6.6 Measurement

A.6.6.1 Micro-plate reader

Pipette a consistent volume of the solution (A.6.5.2) to the well of a microtitre plate (A.4.8) so that the well is almost full, e.g. 490 µl in a 500 µl well. Measure the absorbance versus the blank at a specific wavelength in the range of 600 nm to 750 nm.

NOTE It is important for uniform results that the standard solutions, together with the glove extracts, be analysed together within 1 h after a stable colour has developed.

A.6.6.2 Spectrophotometer

Transfer the solution (A.6.5.2) to a cuvette (A.4.9) and measure the absorbance against the blank at a specific wavelength in the range 600 nm to 760 nm.

NOTE It is important for uniform results that the standard solutions, together with the glove extracts, be analysed together within 1 h after a stable colour has developed.

A.7 Expression of results

A.7.1 Calculation

A.7.1.1 Calibration curve

Calculate the average absorbance of the duplicate determinations. If the individual values differ by more than 20 % repeat the determination. Prepare the calibration curve by plotting average absorbance measurements.

against the true concentration of the original protein standard solutions as shown in Figure A.2. The calibration curve should be linear over the range of 0 µg to 100 µg protein/ml in the original protein standard solutions.

NOTE Some protein is lost during the concentration process. It is assumed that the same percentage of protein is lost from the standards as from the test samples during the concentration process.

A.7.1.2 Concentration of extract

For each of the four extracts, calculate the average absorbance of the duplicate determinations (see A.6.4.1). If the individual values differ by more than 20 % repeat the determination. Determine the concentrations of the extracted samples (*C*) in µg/ml extract by reading them directly from the linear portion of the curve.

NOTE In the event that the calibration curve is non-linear, the value can be calculated by quadratic regression. It is suggested that commercial computer software for curve fitting and calculation of unknown concentrations is more practical.

A.7.2 Results

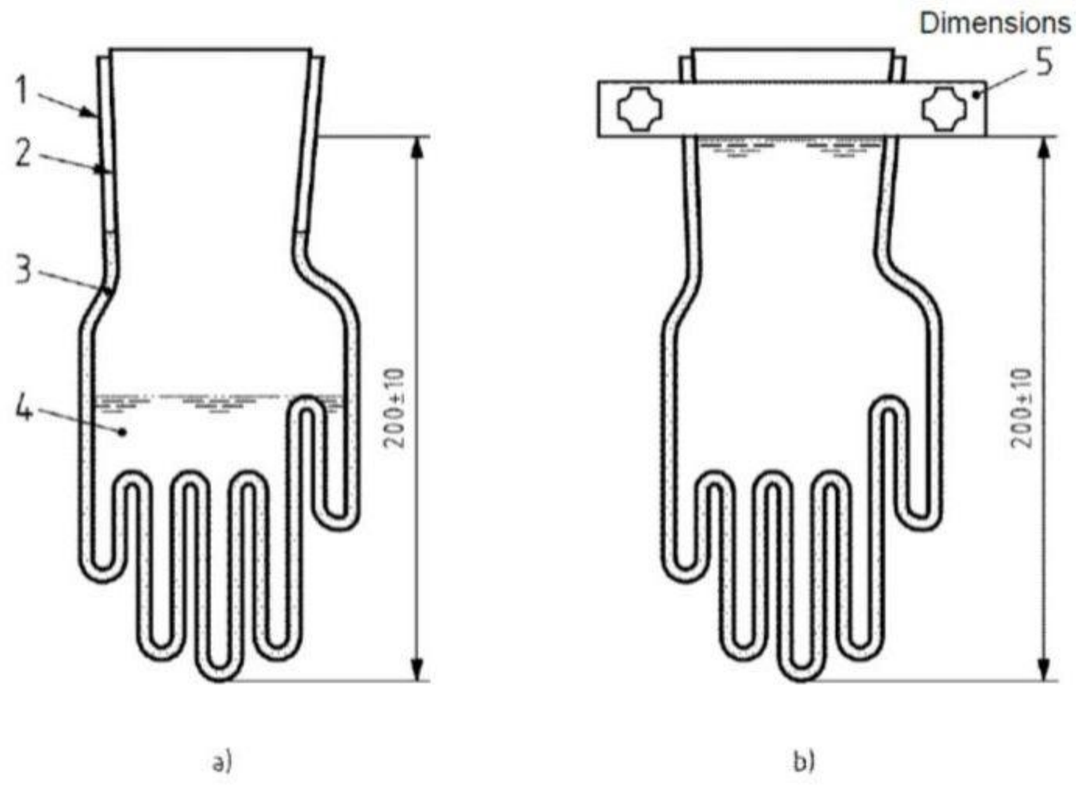
The protein content of each sample is given by:

$$P = \frac{(V \cdot C \cdot F)}{m}$$

where

P	is extractable protein in µg/g of glove;
V	is the volume of extraction medium used in ml;
C	is the protein concentration of the extract in µg/ml;
F	is the dilution factor;
NOTE	F is the real volume of NaOH solution in ml used to re-dissolve the protein divided by 0,2.
m	is the mass of glove extracted in g (A.6.2.6).

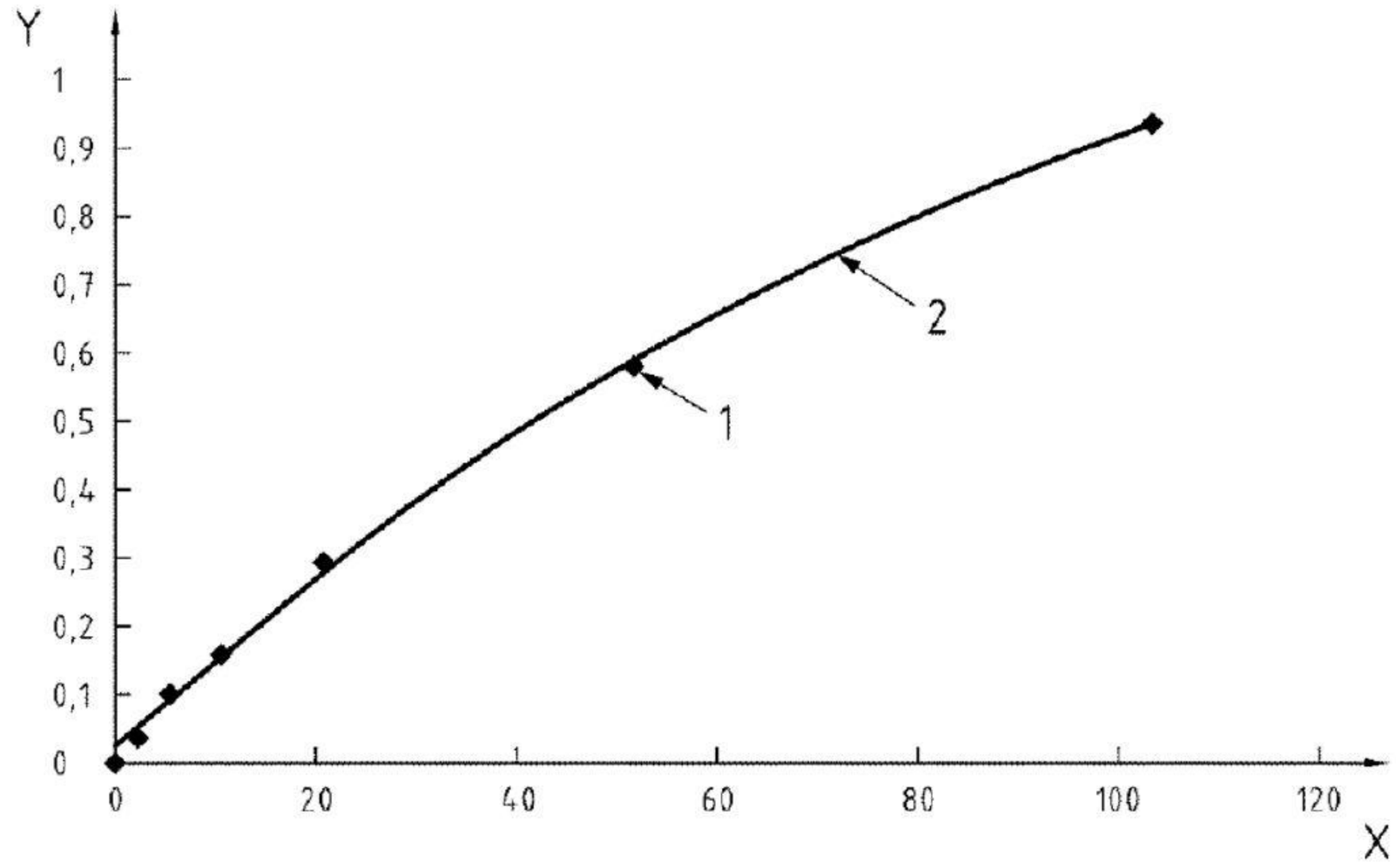
Report the mean protein content of the four determinations of glove extracts.



Key

1 outer glove (glove 1)
2 inner glove (glove 2)
3 extraction buffer
4 dye solution
5 glove clamp

Figure A.1 — Extraction of gloves (cross section)



Key

Y absorbance at 750 nm

X ovalbumin concentration (µg/ml)

1 absorbance

2 computer generated polynoma of best fitting

$Y = -4E - 0{,}5x^2 + 0{,}013x + 0{,}0247$

Concentration [µg/ml]	Absorbance
2,1	0,036
5,2	0,099
10,4	0,159
20,8	0,291
52,0	0,583
104,0	0,945

Figure A.2 — typical standard curve measured in a spectrophotometer at 750 nm with 1 cm path length

A.7.3 Statistical information

Nine laboratories participated in an inter-laboratory exercise as part of a scientific study supported by the EU in 1996 to 1998 and published in the final report MAT 1 – CT 940060 European Commission Directorate General XII. In this experiment both were tested the precision of the Lowry method and the precision of the whole procedure including the extraction. The whole method includes additionally the variation of the protein content from glove to glove, which is in some cases much higher than the variation of the method. The results are summarized in Table A.1.

Table A.1 — Statistical information

	Number of measurements	Number of extracts	Number of days	Mean in µg/ml	Repeatability coeffiecient of variation in % (within laboratories)	Reproducibility coefficient of variation in % (between laboratories)
Glove extract	8 triplicates	1 used by all participants	1	63,9	4,9	9,6
Glove extract	15 triplicates		5	61,7	6,8	6,3
Glove A	5 triplicates	5	1	88,8	7,9	22,5
Glove A	5 triplicates	5	5	84,5	6,1	20,3
Glove B	3 triplicates	3	1	109	20,2	23,3
Glove C	3 triplicates	3	1	727	8,3	23,0
Glove D	3 triplicates	3	1	46,5	10,1	31,8
Mean extract without extraction procedure					5,0	8,0
Mean whole procedure (glove A to D)					10,5	24,2

The limit of quantification was set to 10 µg/g because it is dependent on the thickness (weight) of the gloves. It was found to be between 1 µg/g and 5 µg/g.

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Annex B
(informative)
Immunological methods for the measurements of natural rubber latex allergens

B.1 Introduction

Immediate allergic reactions to natural rubber latex (NRL) proteins are recognized as an important medical and occupational health problem. A major source of sensitisation has been considered to be proteins or peptides eluting from protective NRL gloves [1].

Although the amount of extractable total protein usually correlates reasonably well with the allergen content of NRL gloves measured by skin prick test (SPT) or human IgE-based assays [2], [3], [4], [5], the total protein methods also measure non-allergenic proteins that are unikely to be relevant in NLR allergy. Therefore, there has been an increasing need for methods capable of specifically and accurately measuring allergens in NRL goods. It is agreed that allergen-specific assays would provide much more accurate and reliable information both for regulatory purposes and for monitoring manufacturing processes. The availability of specific assays has, however, been scanty. Moreover, the still incomplete knowledge on the overall significance of the wide spectrum of NRL allergens has made it difficult to decide which of the numerous allergens present in the NRL source material should be measured.

Semiquantitative methods, such as RAST-inhibition and IgE ELISA inhibition, based on the use of human IgE antibodies, have been available for several years in research laboratories. Drawbacks of these methods are that they are difficult to standardise and they suffer from limited availability of human sera containing clinically relevant latex-specific IgE antibodies. In addition, it should be noted that the standards used do not equate to glove proteins. The principle that an ideal test for assessing allergenic potential of NRL products should be based on specific allergen quantification has recently been adopted and endorsed in the ongoing standardisation work both in Europe [6], [7] and the US [8], [31].

Substantial progress has recently been made in the development of specific and quantitative assays for individual NRL allergen quantification [9], [10], [30]. These new assays, based on the capture-enzyme immunoassay (EIA)-principle and on the use of monoclonal antibodies and purified or recombinant allergens, are specific; they can be properly standardized and are of sufficient sensitivity and reproducibility. In this informative annex current methods for NRL allergen measurement are reviewed.

B.2 Natural rubber latex allergens in manufactured rubber products

Of the some 250 different proteins or polypeptides demonstrated in the NRL source material, the liquid latex of the rubber tree, *Hevea brasiliensis*, about one fourth to one fifth have been shown to bind with IgE and represent allergens [11], [12]. The mixture of plant proteins in source material reflects the stress response of the rubber tree to wounding (the tapping procedure). Several of these proteins are defense proteins that have been well preserved in plants during evolution. The structural homologies shared with these proteins provide the molecular basis for the common cross-reactions of latex-allergic patients' IgE towards various plant proteins. It is likely that all of the significant allergens probably are present in the liquid NRL but, as referred to above, the majority of proteins and polypeptides present in the NRL source material are likely to be irrelevant in the assessment of allergenic properties of manufactured NRL products. The WHO/IUIS Allergen Nomenclature Committee lists

(February 2013) 14 NRL allergens characterized t the molecular level (www.allergen.org.), most of which have been cloned and produced by recombinant DNA techniques.
An optimal test should be designed to accurately measure all allergens that can be present in manufactured rubber products. This could include epitopes present on the natural proteins, as well as new epitopes on the break down products resulting from the harsh rubber manufacturing processes. So far a limited number of Hev b 1, Hev b 3, Hev b 5 and Hev b 6.02, and/or fragments or polymers of them carrying IgE-binding epitopes, can be present in manufactured products [13], [14], [15], [16], [17], [18]. Whether additional allergens, such as Hev b2, Hev b7 Hev b13 [19] or Hev b14 [32], turn out to be important rubber product-specific allergens, still awaits confirmation.

B.3 Methods for measuring natural rubber latex allergens

B.3.1 Qualitative methods

Immunoelectrophoretic methods and immunoblotting techniques, used extensively in the early 1990s, demonstrated and tentatively characterised several NRL proteins to which IgE from sera of NRL-allergic patients bind. It is however today agreed that these methods alone cannot be used for reliable identification of allergens [11], [12], [20], [21].

B.3.2 Semiquantitative methods

B.3.2.1 Skin prick testing in voluntary latex-allergic subjects

Allergenicity of NRL extracts can be semiquantitatively assessed by skin prick testing (SPT) in a statistically relevant number of NRL-allergic patients. The size of the reaction is dependent on and proportional to the quantity of the allergens to which the patient has IgE class antibodies [**2**]. From a biological point of view SPT would make an ideal test to assess clinically relevant allergenicity, but due to ethical constraints this approach cannot be routinely used as a test for monitoring allergen contents in NRL gloves.

B.3.2.2 IgE-ELISA inhibition (also known as RAST-Inhibition)

ELISA-inhibition (ELISA = enzyme linked immunosorbent assay) can be used on the basis of commercially available or self-made assays for the determination of specific IgE antibodies. The formerly usual RAST (radio allegro sorbent test) used radioactive labelled instead of enzyme labelled detection antibodies.

ELISA inhibition has been used to evaluate NRL allergens in various medical and consumer products [3], [4], [22], [23].

In the procedure, optimal amounts of NRL allergens are bound to solid phase (e.g. paper or polystyrene). Unknown samples and standards are incubated with pooled IgE serum from individuals with confirmed NRL allergy. When the IgE antibody binds to the soluble allergen, it is prevented from binding to the solid phase allergen. After incubation the mixture is transferred to the immobilised allergen preparation where the free IgE- antibodies are bound to the allergens on the solid phase. Specific binding is then measured using an enzyme labelled anti-IgE antibody. The extent of inhibition is proportional to the quantity of soluble allergens in the extract.

The critical reagents are the immobilised allergen, the human serum pool and the standard allergen.

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In the self-made assay used in reference 4 native non ammoniated NRL was used for coating and as standard allergen. A concentration of 10 mg protein per ml in the standard was set to 100 000 arbitrary units. Serial dilutions of glove extracts and standard NRL dilutions are incubated with optimally diluted IgE serum pool composed of carefully characterised high-titered sera from NRL- allergic patients [4].

B.3.3 Specific quantitative methods

B.3.3.1 Capture enzyme immunoassays (EIA) for NRL allergen quantification

B.3.3.2 Background

The principle has been acknowledged that an optimal assay should be designed to detect only those NRL allergens that have been shown to be present in manufactured products. Four NRL allergens, i.e. Hev b 1,

gloves [13], [15], [16], [17], [24]. The two most important allergens for adult subjects are Hev b 5 and Hev b 6.02 (hevein) [15], [17], [25]. Hev b 1 and Hev b 3 are important allergens for children with spina bifida [26], [27]. Allergen-specific capture-enzyme immunoassays (EIA) to quantify these four NRL allergens have recently been developed and kits to measure these allergens have been commercially available since December 2001. Reagents and equipment can also be purchased separately.

B.3.3.3 Description of capture EIA methods [4)]

The capture EIAs make use of specific monoclonal antibodies and either purified allergens or proteins produced by recombinant DNA technology as standards. Microtitre wells are coated in each test with one specific monoclonal antibody that binds the desired allergen from the sample. After incubation, unbound material is removed by washing. In the second incubation, enzyme-labelled (usually horseradish peroxidase (HRP)) allergen-specific monoclonal antibody binds to allergen molecules bound on the microtitre plate in the first incubation. After washing, substrate for the enzyme is added. After stopping the reaction the absorbance at a suitable wavelength is measured. The intensity of the colour produced is directly proportional to the allergen concentration of the sample.

B.3.3.4 Performance of the capture EIAs in comparison with IgE-based allergen assays

Specific allergen tests have now been used in several series of studies to assess the allergenicity of medical gloves. The best verification of the allergenic potential of a given extract would be reflected in its reactivity in the skin of NRL-allergic patients. In a study of 22 NRL gloves highly significant correlations emerged when the sum of these 4 allergens (measured by the commercial capture EIA kit) was related to results from human IgE-based inhibition assays [10]. Highest correlation was seen between the sum of the 4 allergens in the gloves and SPT in 20 NRL allergic volunteers (r = 0,95) followed by the sum and IgE ELISA inhibition results (r = 0,90). The correlation to total protein measured with the Modified Lowry method was very low (r= -0,11). In another series of 58 NRL gloves reported in the same communication [10] the correlation between the sum of the four allergens and total allergen activity by IgE ELISA inhibition was 0,84. Results of a recent international multicenter study [28] arranged by FDA and carried out in seven laboratories to measure NRL allergens in 30

4) According to information provided by the manufacturer of the commercially available kits (FITkit□ Insert leaflets, www.quattromed.com) the limit of detection for the 4 allergens range from 0.1 µg/l (Hev b6.02) to 2,3 µg/l (Hev b3). Repeatability coefficient of variation has been shown to range from 2,8 % to 5,8 %, and reproducibility coefficient of variation from 2,6 % to 7,6 %. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named.

gloves similarly showed that sum of the four allergens measured by the monoclonal EIAs displayed the highest correlation (r2 = 0,91 – 0,95) with the assays using human IgE-based RAST/ELISA inhibition. For the time being, extended studies with a large number of gloves are still needed to further assure the applicability of the allergen-specific EIAs but it already appears that the sum of the four allergens reflects the total allergenic content of the glove extracts in a biologically meaningful manner. Studies currently in progress are expected to enlighten whether additional allergens are needed and whether they would affect the outcome of the assessments.

Another study of 208 gloves [30] using the commercial immunoassay (FitKit, Icosagen AS, Tartu, Estonia) fulfilling the ASTM D7427-08 Standard criteria provided strong correlation between the sum of four clinically relevant allergens and Skin Prick Test-validated human IgE –based immunoassay [4]. For the time being, extended studies with a larger number of gloves are still needed to further assure the applicability of the allergen-specific EIAs but it already-appears that the sum of the four allergens reflects the total allergenic content of the glove extracts in a biologically meaningful manner even though no claims of safety can be presented. Further studies currently in progress are needed to enlighten whether additional allergens are needed and whether they would affect the outcome of the assessments.

B.4 Conclusion

Measuring total extractable proteins is not deemed an ideal method to control NRL allergen content of medical gloves. However, at the time of the publication of the standard, specific human IgE based methods for measuring allergens are not validated, lack standardization and suffer from paucity of the required reagents. Therefore, it is still the method prescribed in the normative part of this standard. Capture EIA methods for the quantification of NRL allergens overcome several of the limitations of previous methods by using characterised and highly purified allergens and specific monoclonal antibodies against NRL allergens known to be present in NRL products. The assays have high specificity, irrespective of presence of other proteins or chemical substances derived from the manufacturing process of NRL products, and high sensitivity. The tests are technically relatively easy to perform and the results can be obtained in a short assay time (< 2 h). Drawbacks include the currently high costs and that it has not yet been possible to settle with certainty which of the several known NRL allergens are needed to establish recommendations and safety limits. Also a large number of monoclonal antibodies may be needed to ensure detection of all relevant allergens. Currently, tests and/or reagents for measuring four individual NRL allergens by capture-EIA-based methods are commercially available. Should additional allergens turn out to occur in significant amounts in rubber products, new reagents and kits based on the existing frameworks can be developed.
An interlaboratory experiment regarding the assessment of three test methods dealing with the quantification of NRL proteins and allergens in medical gloves was carried out by CEN/TC205/WG3 in the year 2002. The three test methods evaluated were

— Measurement of specific allergens (see footnote 4 from B.3.3.3)
— ASTM D 6499 (antigenic proteins) [29]
— Amino acid analysis (total protein)

No conclusions could be drawn from this experiment allowing to recommend the above mentioned test methods to be introduced as normative into the standard.
Extended studies with a representative collection of gloves currently marketed in Europe and reference samples with accurately quantified concentrations of allergens of interest are needed to further validate the performance and usefulness of the new allergen-specific assays.

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Annex C
(informative)
Amino acid analysis (AAA) by high pressure liquid chromatography (HPLC)

C.1 Background

The determination of proteins is usually based on colour reactions with particular structural elements, which are not regularly distributed in different proteins [1], [2], [3], [4], [5]. Therefore the response factor considerably differs from protein to protein [2], [4]. In addition a number of substances interfere with colorimetric assays due to either unspecific reactions with the colour reagent or inhibition of the colour development.

The amino acid analysis avoids these problems. This was confirmed by the results of the study 'Determination of allergological relevant compounds in disposable gloves - Correlation of chemical, allergological and immunological data' within the program 'Measurement and Testing' of the European Commission (MATI-CT 940060) [8]. In this study the best correlation between clinical data (prick test) and the chemical analysis was revealed, if the protein concentration was measured by the amino acid analysis [6].

Nevertheless the modified Lowry method should be used as the standard method for protein determination in natural rubber gloves, because the amino acid analysis seemed to be too uncommon and complex for a standard procedure. The amino acid analysis may be used to clarify doubtful results revealed by the modified Lowry method. It should not be used for protein declaration but should help the manufacturer to avoid substances that lead to incorrect protein determination by the standard method.

C.2 Principles of the determination of proteins by HPLC

In the first step the proteins are hydrolysed by 6 *M* hydrochloric acid. The resulting free amino acids are then separated and detected by HPLC [7]. Quantification through an internal standard (norvalin) and subsequent summation of the individual amino acids reveal the total protein content. Due to this procedure the method is independent from any structural feature of the original polymeric molecule. Currently no interfering substances could be found, but the presence of TES-salts seem to avoid the loss of amino acids (e.g. by wall effects).

C.3 Material

C.3.1 DL-Norvalin.

C.3.2 HCl 30 % Suprapur.

C.3.3 Amino acid standard (containing L-alanine, ammonium chloride, L-arginine, L-aspartic acid, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methio-nine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine 0,5 mM each and L-cystine 0,25 mM).

C.3.4 Methanol protein sequencing grade.

C.3.5 o-Phthaldialdehyde (OPA).

C.3.6 Boric acid.

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C.3.7 Ethylendiaminetetratacetic acid, disodium salt (EDTA).

C.3.8 Potassium phosphate monobasic (KH2PO4).

C.3.9 Sodium phosphate dibasic (Na2HPO4).

C.3.10 Sodium phosphate monobasic (NaH2PO4).

C.3.11 3-mercaptopropionic acid.

C.3.12 Separation column: Hypersil ODS 3 µm, 150 x 4,6 mm, pre-tested for OPA application.

C.3.13 Precolumn: Hypersil ODS, 3 µm, 5 x 4,6 mm.

C.3.14 Water at least Milli-Q or equivalent quality.

C.3.15 Filter unit 0,2 µm pore size.

C.3.16 Tetrahydrofuran (THF) gradient grade for liquid chromatography.

C.3.17 Acetonitril gradient grade for liquid chromatography.

C.3.18 2 ml screw capped polypropylene vessels.

C.3.19 Sodium carbonate.

C.3.20 Sodium hydroxide or potassium hydroxide pellets.

C.4 Buffers and solutions

NOTE Solvent 1 and solvent 2 are made for an OPA-1 column of Grom, Herrenberg, Germany. If any other columns are used changes might be necessary.

C.4.1 Norvalin-100

11,7 mg norvalin (C.3.1) in 1 ml water (C.3.14) = 100 mM norvalin.

C.4.2 Norvalin-1

100 µl norvalin-100 (C.4.1) in 10 ml water (C.3.14) = 1 mM norvalin, store at below 8 °C not longer than 4 weeks.

C.4.3 o-Phthaldialdehyde (OPA)

50 mg o-phthaldialdehyde (C.3.5), 4,5 ml methanol (C.3.4), 50 µl mercaptopropionic acid (C.3.11).

C.4.4 Boratebuffer

400 mM sodium borate, 5 mM EDTA, pH 10,4.

1,24 g boric acid and 85 mg EDTA in 30 ml water (C.3.14), adjust to pH 10,4 with 2 M NaOH and add water (C.3.14) ad 50 ml. Filter through a 0,2 µm filter unit (C.3.15), store at room temperature not longer than two weeks. Avoid refrigeration which causes an insoluble precipitate.

C.4.5 Stop-solution

1,36 g KH_2PO_4 (C.3.8) in water (C.3.14), filter through 0,2 µm filter unit (C.3.15) and store at room temperature not longer than 4 weeks.

C.4.6 Phosphate buffer

7,15 g Na_2HPO_4 (C.3.8) and 3,45 g NaH_2PO_4-H_2O (C.3.9) in 1,5 l water (C.3.14).

C.4.7 Solvent 1

20 ml tetrahydrofuran (C.3.16) plus 1 l phosphate buffer (C.4.6).

C.4.8 Solvent 2

250 ml acetonitril (C.3.17) 100 ml tertrahydrofuran (C.3.16) ad 1 l with phosphate buffer (C.4.6).

C.4.9 Sodium carbonate solution (0,1 M)

2,12 g Sodium carbonate (C.3.19) in 10 ml water (C.3.14).

C.5 Hydrolysis

C.5.1 Samples

400 µl extract (in TES buffer) + 10 µl norvalin-1 (C.4.2) + 700 µl HCl (C.3.3).

C.5.2 Standards

380 µl water (C.3.14) + 20 µl amino acid standard (C.3.3) + 10 µl norvalin-1 (C.4.2) + 700 µl HCl (C.3.2).

C.5.3 Incubation (hydrolysis)

Incubate samples and standards simultaneously for 48 h at 100 °C in sealed screw capped PP vessels (C.3.18). The vessels should be clamped into a screwed rack to avoid cracking of the caps. It is very important to hydrolyse standards and samples simultaneously in order to have equal temperature and time conditions.

Cool down samples and standards, dry them in vacuum concentrator centrifuge or in a desiccator over NaOH or KOH in vacuum.

HCl need to be removed completely; otherwise the capacity of the borate buffer for derivatisation may be not sufficient.

C.5.4 Free amino acids

Prepare from each extract and from the standard an un-hydrolysed sample.
— 400 µl extract + 10 µl norvalin 1 (C.4.2);
— 380 µl water (C.3.14) + 20 µl amino acid standard (C.3.3) + 10 µl norvalin 1 (C.4.2).

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C.6 Analysis (HPLC)

C.6.1 Sample preparation

— Add 20 µl sodium carbonate solution to the dried samples (C.4.9);
— mix well or sonicate;
— incubate for 15 min at room temperature, mix again to remove CO2;
— add 180 µl borate buffer (C.4.4).

C.6.2 Derivatisation

The derivatisation step is dependent on time and temperature; it should be done by an autosampler at constant temperature between 20 °C and 25 °C.

Mix 25 µl borate buffer (C.4.4), 12 µl OPA (C.4.3) and 8 µl sample.

After 2,5 min terminate the reaction by adding 25 µl stop solution (C.4.5).

C.6.3 HPLC

The HPLC analysis can be done in any HPLC equipment using a gradient system and fluorescence detector

A successful example is listed here, but these conditions have to be adapted to the system and the column used.

EXAMPLE

0 min to 2,5 min	0 % solvent 2	100 % solvent 1
2,5 min to 3,0 min	0 % to 12,5% solvent 2	87,5 % to 100 % solvent 1
3,0 min to 9,0 min	12,5 % solvent 2	87,5 % solvent 1
9,0 min to 13,0 min	12,5 % to 42% solvent 2	58 % to 87,5 % solvent 1
13,0 min to 24,0 min	42 % solvent 2	58 % solvent 1
24,0 min to 26,0 min	42 % to 80% solvent 2	20 % to 58 % solvent 1
26,0 min to 30,0 min	80 % solvent 2	20 % solvent 1
30,0 min to 31,0 min	0 % to 80% solvent 2	20 % to 100 % solvent 1

C.6.4 Calculation

The concentration of the individual amino acids shall be performed by an internal standard method with subtraction of the free amino acids. The sum of the amino acids equals the total protein content.

C.7 Examples

C.7.1 Standard

A typical chromatogram of a hydrolysed standard solution with 19 amino acids in equimolar concentrations is shown in Figure C.1 a). The expected amino acids are listed in Table C.1. Asparagine and glutamine which completely convert to aspartic acid and glutamic acid were not included in the standard solution. Norvaline which does not occur naturally was used as an internal standard. Tryptophan and cystine were present in the unhydrolyzed standard solution but were destroyed by HCl-hydrolysis. Proline does not react with OPA/MPA due to the lack of a primary amino group and therefore was not detectable under these derivatisation conditions. Lysine often results in two peaks because one or two of its amino groups may react with OPA/MPA. The ratio of these two peaks is dependent on the reaction conditions

(temperature, age of OPA solution) and therefore varies from run to run but does not influence the results if both peak areas are taken into account.

C.7.2 Glove extract

The chromatogram of a hydrolysed glove extract (prepared as described in Annex A) is shown in Figure C.1 b). This hydrolysis of latex proteins revealed the complete panel of the expected amino acids (Table C.1). Additional peaks were found at 14,23 min and 24,08 min which were identified as TES derived products. This peak was completely resolved from those of all amino acids and did not influence the analysis.

C.8 Advantages and disadvantages of the HPLC method

C.8.1 Advantages

— It is not dependent on the polymeric structure of the protein;
— It revealed the best correlation to clinical data (prick test);
— No interfering substances are known;
— It is more sensitive than colorimetric determinations;
— It is highly specific for proteins.

C.8.2 Disadvantages

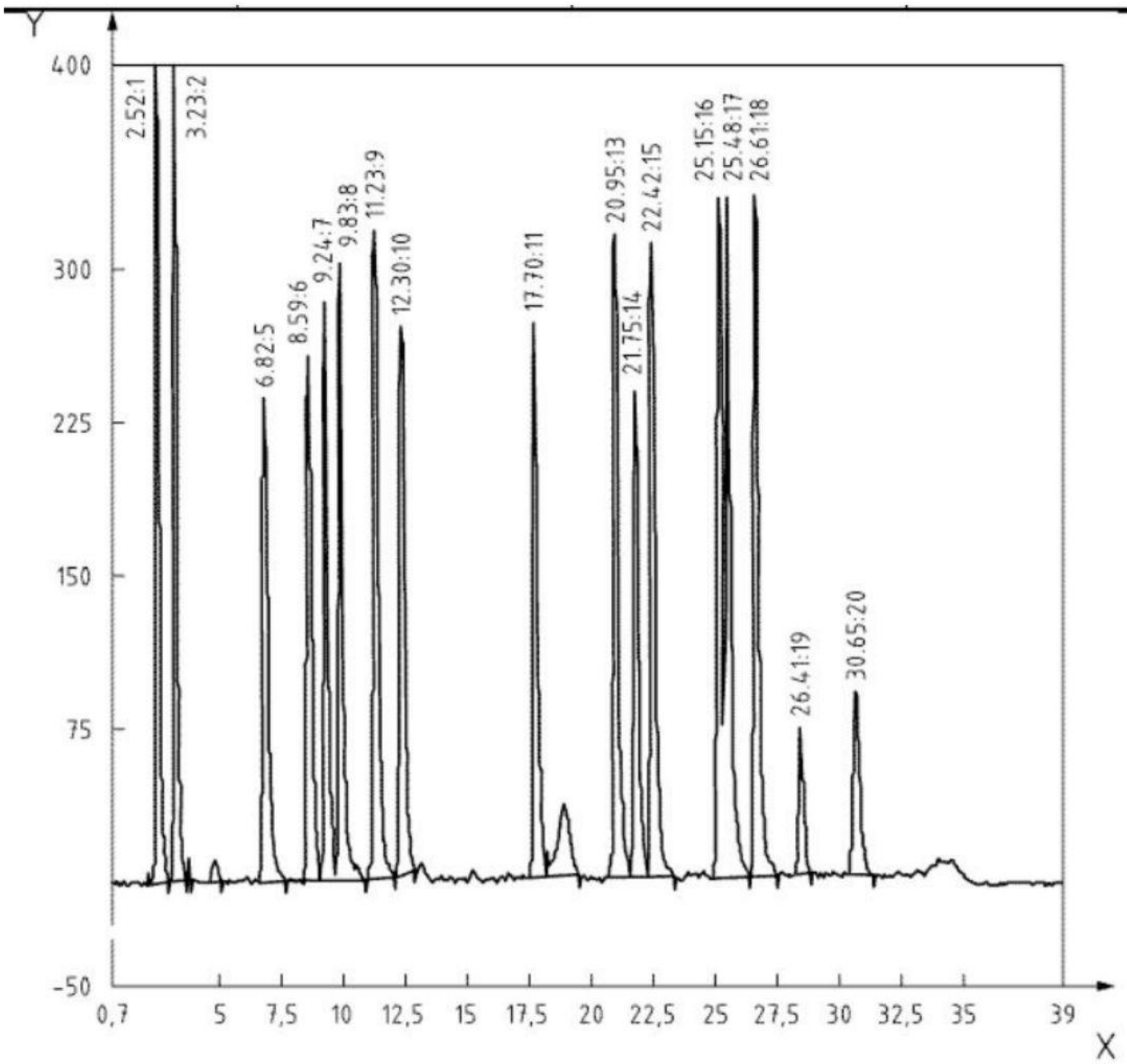
— It is an uncommon method, installed only in a few laboratories.
— It is time consuming.
— The very complex evaluation of the data requires a lot of experience.

Table C.1 — List of amino acids found in the HPLC analysis of a standard solution (Figure C.1 a)) and in the hydrolysis of the glove extract (Figure C.1 b))

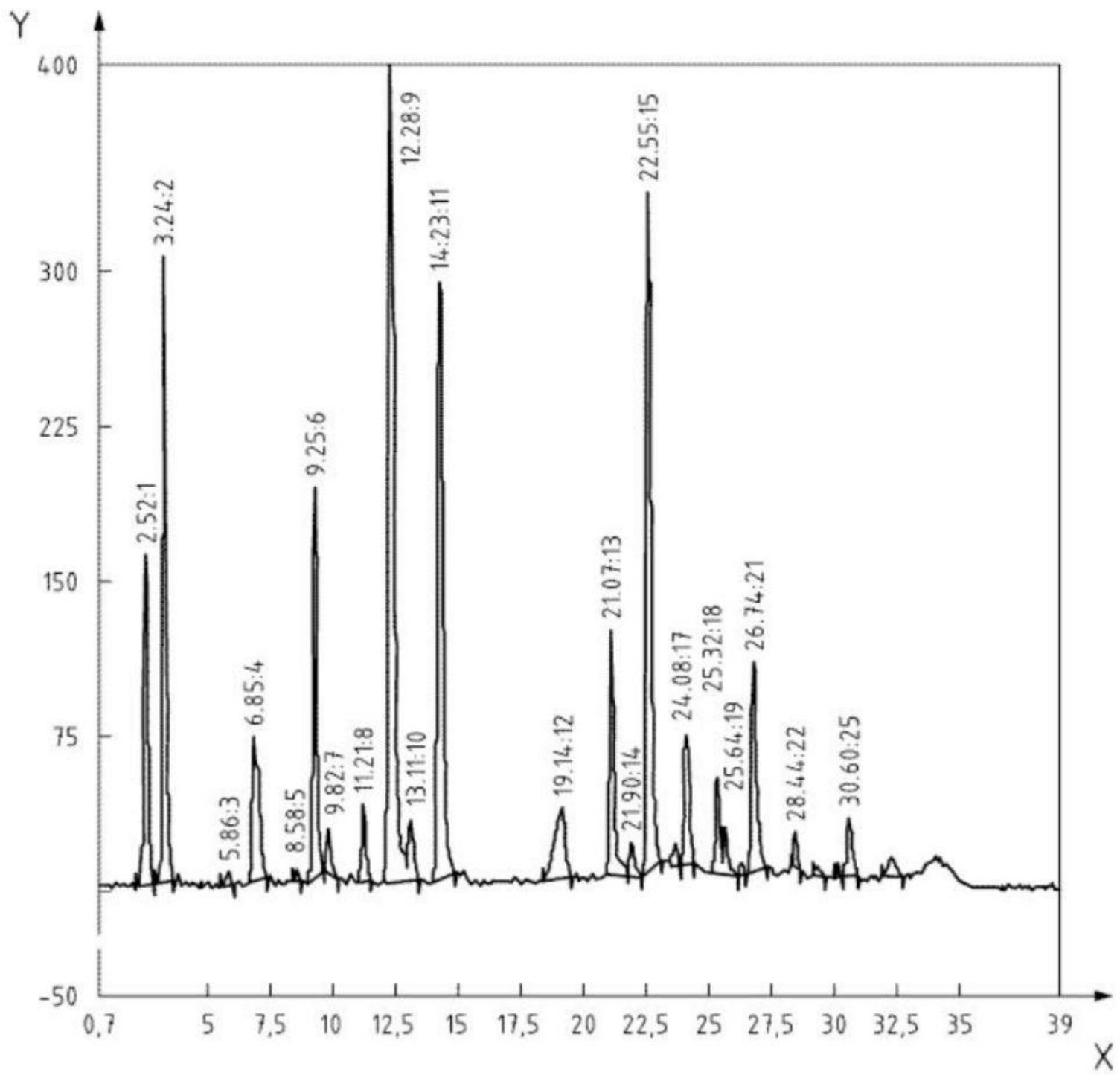
Amino acid	Retention time in min		Comment
	Standard	Analysis	
Aspartic acid (ASP)	2,52	2,52	
Asparagine (ASN)			converted to ASP
Glutamic acid (GLU)	3,23	3,24	
Glutamine (GLN)			converted to GLU
Serine (SER)	6,83	6,85	
Histidine (HIS)	8,60		
Glycine (GLY)	9,25	9,25	
Threonine (THR)	9,84	9,82	
Arginine (ARG)	11,24	11,21	
Alanine (ALA)	12,30	12,29	
		14,23	TES (extraction buffer)
Tyrosine (TYR)	17,7		
Valine (VAL)	20,95	21,07	
Methionine (MET)	21,75	21,90	
Norvaline (NORVAL)	22,42	22,55	internal standard
		24,08	TES (extraction buffer)
iso-Leucine (ILE)	25,15	25,32	
Phenylalanine (PHE)	25,48	25,64	
Leucine (LEU)	26,61	26,74	

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Lysine (LYS)	28,41 30,65	28,44 30,60	
Tryptophan (TRY)			destroyed by hydrolysis
Cystine, Cysteine (CYS)			destroyed by hydrolysis
Proline (PRO)			not detectable



a) Amino acid standard



b) Glove extract

Figure C.1 — typical chromatograms of an amino acid standard (A) and an analysis of a glove extract (35 µg protein)

C.9 References

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[8] MATI_CT 940064 European Commission Study - Determination of allergological relevant compounds in disposable gloves - Correlation of chemical allergological and immunological data

Annex ZA
(informative)
Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC Medical Devices

This Standard has been prepared under a mandate given to CEN by the European Union and the European Free Trade Association to provide a means of conforming to Essential Requirements of the New Approach Directive 93/42/EEC Medical Devices.

Once this standard is cited in the Official Journal of the European Union under that Directive and has been implemented as a national standard in at least one Member State, compliance with the clauses of this standard given in Table ZA.1 confers, within the limits of the scope of this standard, a presumption of conformity with the corresponding Essential Requirements of that Directive and associated EFTA regulations.

Table ZA.1 — Correspondence between this European Standard and Directive 93/42/EEC Medical Devices

Clause(s)/subclause(s) of this EN	Essential Requirements (ERs) of Directive 93/42/EEC Medical Devices	Qualifying remarks/Notes
4	6, 7.1,7.2,7.5	
4.6	2, 13.1,13.3	

For devices intended by the manufacturer to be for dual use in accordance with Article 1(6) of Directive 93/42 EEC the following Table ZA.2 details the relevant essential requirements of Directive 89/686/EEC on Personal Protective Equipment and their corresponding clauses of this European Standard. Table ZA.2 however, does not imply any citation in the OJEU under the PPE directive and thus does not provide presumption of conformity for the PPE directive.

Table ZA.2 — Relevant Essential Requirements from Directive 89/686/EEC on Personal Protective Equipment that are addressed by this European Standard

Clause(s)/subclause(s) of this EN	Essential Requirements (ERs) of Directive 89/686/EEC on Personal Protective Equipment	Qualifying remarks/Notes
4.2	1.2.1.1.	

WARNING — Other requirements and other EU Directives may be applicable to the product(s) falling within the scope of this standard.

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Informasi pendukung terkait perumus standar

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Komite Teknis 13-09 Biosafety and Biosecurity

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